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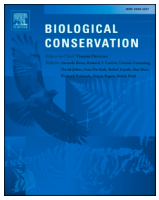

Unlocking the genetic enigma: A paradigm shift towards evidence-based conservation of Indian star tortoise (*Geochelone elegans*)

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ABSTRACT

Management and conservation of highly trafficked species suffer from a lack of baseline data on their status in the wild. This study provides insights into the historical drivers shaping contemporary genetic variation in the world's most trafficked turtle, the Indian star tortoise (IST), *Geochelone elegans*. We employed an integrative approach combining two mtDNA regions, six polymorphic microsatellites, and morphometry to characterize wild ISTs covering the entire range of the species. Contrary to expectations and despite decades of illegal poaching and unscientific release, the results reveal two major clusters in the Indian subcontinent: a relatively invariable northwestern group (Kutch and Saurashtra in Gujarat, Malwa plateau and Aravali in Rajasthan) and a highly diverse southern group (Eastern ghats, Deccan plateau, Nilgiri hills, Coromandel coast, and Sri Lanka). Contradicting most previous studies, the species exhibits high genetic diversity, especially the southern group, a genetic hotspot with multiple sub-clusters inhabiting a widely reported poaching hotspot. The two deeply divergent groups (northwestern and southern) split around 2 Ma during Pleistocene and should be considered separate subspecies (northwestern *Geochelone elegans stellata* and southern *Geochelone elegans elegans*). Reconstruction of historical demography suggests a stable historical population with a recent declining trend over last 100–120 years. Our study presents a comprehensive reference database that can be used for scientific release and rehabilitation of seizures and guide effective management and conservation. Integrating genetic intelligence into management of highly trafficked species will aid in shifting current adhocism towards evidence-based conservation, leveraging the species' biological traits and natural genetic variation.

1. Introduction

The megadiverse illegal wildlife trafficking (IWT) of >100 million plants and animals annually is associated with striking declines in the abundance of highly trafficked species (HTS) ('t Sas-Rolfes et al., 2019; Morton et al., 2021; Cardoso et al., 2021). Addressing this global policy and conservation challenge requires convergence of theoretical and practical evidence to clearly understand its drivers and impacts across temporal and spatial levels, involving multi-sectoral stakeholders ('t Sas-Rolfes et al., 2019; Cardoso et al., 2021). The massive scale of collections and mostly unscientific translocations in HTS not only hamper natural recruitment but also enable the loss of locally adapted genetic variations (Amos and Balmford, 2001; Frankham, 2005; Alacs et al., 2007; CITES, 2019). The management of HTS is further compounded by ad hoc strategies that may be inadequate, unscientific, or loosely enforced (Fukushima et al., 2021). Management and conservation policies, unless grounded in scientific evidence with comprehensive geographic coverage, can mislead policymakers and managers, misdirect management strategies, and impede biodiversity conservation ('t Sas-Rolfes et al., 2019; Natusch et al., 2021). Despite the urgency in the face of mounting seizures, a chronic lack of scientific data on the status of HTS in the wild remains a persistent and critical management deficit (Marshall et al., 2020; Morton et al., 2021).

Highly publicized in seizures and popular in IWT, HTS face a double burden of general biodiversity threats such as habitat degradation, climate change, and demographic stochasticity, aggravated by targeted threats such as over-collection for the trade of body parts and pet trade (Auliya et al., 2016). This study focuses on the genetic structure, diversity, and historic demography of the world's most trafficked turtle, the Indian star tortoise (ISTs), which represents around 11 % of global Chelonian seizures (van Dijk, pers. comms. 2016) and 50 % of all turtles seized in India (Traffic, 2019). The IST is endemic to the Indian

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subcontinent, where it occurs in pockets of aridity across two disjunct areas, in the northwest (northwestern India and bordering Pakistan) and south (southern India and Sri Lanka). The two groups exhibit notable morphological differences with a decreasing size gradient from north to south in India, which is reversed in Sri Lanka (Das, 1995; Ernst et al., 1989; de Silva et al., 2017). Owing to the increasing decline across its native range and a corresponding increase in seizures across the world, the IST has been accorded high protection in national legislations across its native states (Schedule I of the Wild life (Protection) Amendment Act, 2022 in India; Schedule II of the Sindh Wildlife Protection Ordinance 1972; and the Sri Lanka Fauna and Flora Ordinance, 1993), classified 'Vulnerable' in the IUCN Red List, and recently included in Appendix I of CITES.

The legislative efforts at national and international levels have not

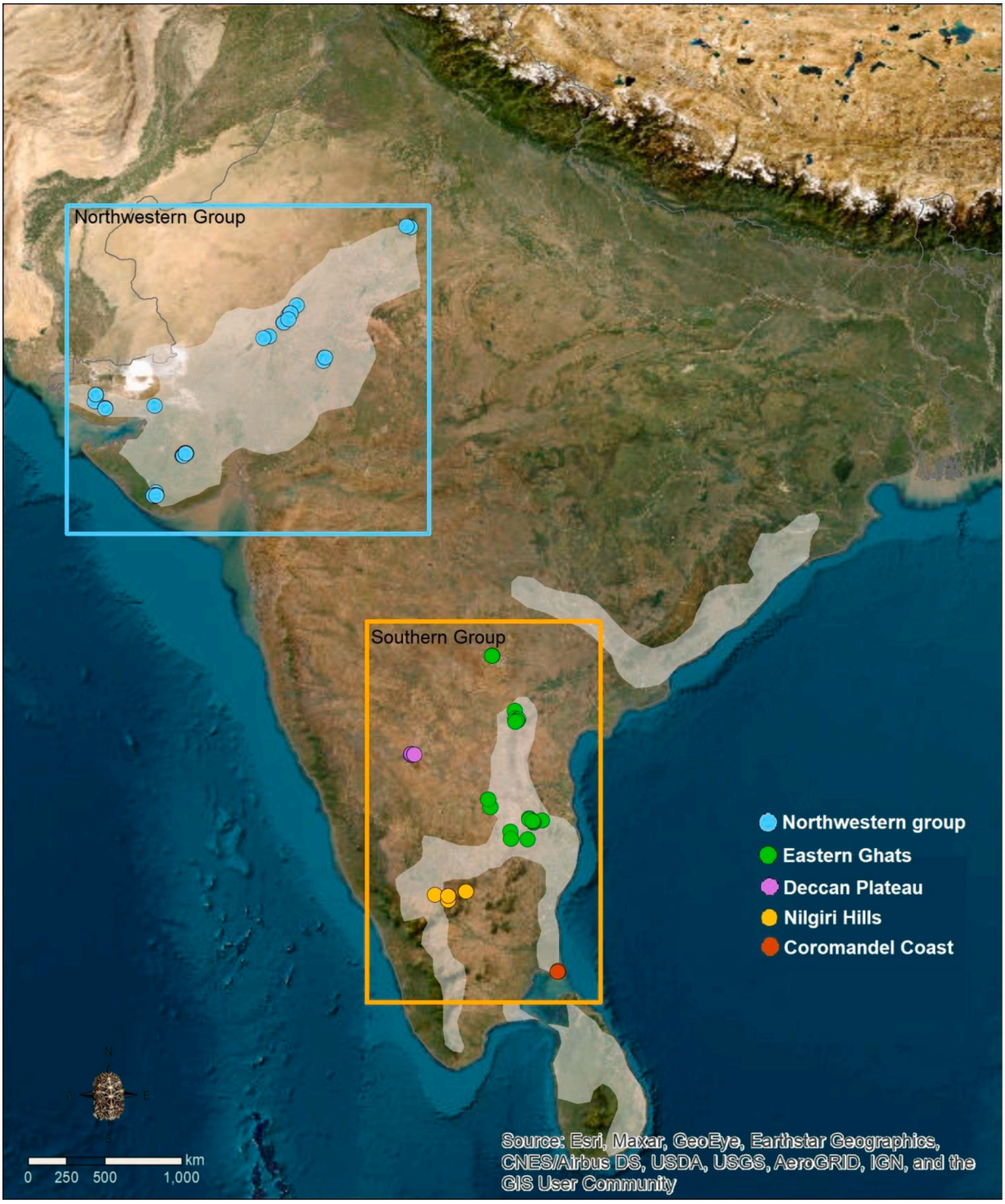

Fig. 1. Sampling sites of *Geochelone elegans* (details in Table S1) collected in this study. Sampling locations corresponding to landscapes are denoted by different coloured dots: Northwestern India (NG) (Blue), Eastern Ghats (EG) (Green), Deccan Peninsula (DP) (Purple), Nilgiri Hills (NH) (Yellow) and Coromandel Coast (CC) (Red). The larger northern and southern distribution ranges are indicated by coloured squares: Northwestern India (Blue) and Southern India (Orange). The shaded areas represent the IUCN distribution range of the species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

been accompanied by on-ground conservation strategies in post-seizure management and monitoring. Though the species has been recorded in several protected areas, the highly fragmented distribution lacks systematic data on its population status in the wild. A comprehensive genetic study can alleviate the twin problems of over-harvesting from the wild and scientific management of increasing seizures through reintroduction, breeding programs, and rehabilitation (Auliya et al., 2016). The prevailing translocation and release practices lack a substantial genetic basis. These decisions are frequently based on impromptu considerations, such as the proximity of forests to the seizure sites, leading to the introduction of confiscated tortoises (purportedly collected from mixed populations) into habitats that are both non-indigenous and unsuitable (Gaur et al., 2006). The pioneering study by Gaur et al. (2006) to determine the origin of confiscated ISTs in Singapore indicated strong genetic structuring in India. Due to limited sampling and genetic characterization based only on captive specimens, the study could not assign provenance for more than half the individuals, highlighting the need for comprehensive sampling in managing HTS (Alacs et al., 2007). Another study by Vamberger et al. (2020) based on captive specimens from all range countries suggested erosion of natural phylogeographic differentiation due to unscientific release and translocation. Recently, Kundu et al. (2022) found diminished genetic differentiation and a decline in genetic diversity among ISTs based on samples sourced from pet stores.

There is an imminent risk of admixture with not only individuals from other geographies but also from the pet trade (Auliya et al., 2016; Stoner and Shepherd, 2020). Multiple natural habitats in India, such as Sathyamangalam Tiger Reserve (TR) and Chinnar Wildlife Sanctuary (WLS), have been turned into release sites, where the impact of admixture on the native population is unknown (Shaji, 2012; Pereira, 2017). This warrants an urgent comprehensive mapping of the genetic variation in the wild to provide a scientific basis for the rehabilitation of confiscated tortoises (Vamberger et al., 2020; Auliya et al., 2016; CITES, 2019), aided by a taxonomic review of genetic differentiation among the various populations (Gaur et al., 2006; Fife, 2007).

This study aims to utilize genetic knowledge to streamline conservation efforts through evidence-based management. Our study objectives were to examine whether the northwestern and southern groups are different, estimate the genetic diversity and differentiation between the groups, infer historical events shaping current distribution, and provide a robust scientific basis for rehabilitating and managing confiscated ISTs. We discuss our findings in the broader context of molecular evidence guiding management practices in the highly trafficked species, rationalizing conservation with resource optimization.

2. Material and methods

2.1. Sampling and DNA extraction

We collected 82 tissue samples from 14 sampling locations, covering the entire range of the IST in India: 38 from northwestern India and 44 from southern India (Fig. 1, Table S1). For each tortoise, approximately 0.5–1 g of keratin scutes layers were peeled off from hind limbs and stored in 90 % ethanol. Genomic DNA was extracted from tissue samples using the QIAGEN DNeasy Blood & Tissue Kit according to the manual instructions and checked by 0.8 % agarose gel electrophoresis.

2.2. PCR amplification, sequencing, and genotyping

Two mitochondrial genes: Cytochrome *b* (Cyt *b*) and NADH dehydrogenase 4 (ND 4) with adjacent Histidine tRNAs were amplified (Gaur et al., 2006; Vamberger et al., 2020) in 20 μl volume, comprising 1 μl template DNA (~20–80 ng), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 3 pmol of each primer, 0.5 units of DreamTaq DNA Polymerase, and the rest distilled H_2O (Thermo Fisher Scientific, MA, USA). The PCR was run under the following conditions: Initial denaturation for 5 min at 95 °C, 35 cycles of 45 s at 95 °C, annealing for 60 s at 56 °C and 75 s at 72 °C, and a final extension for 10 min at 72 °C. All the PCR experiments included negative controls and the products were checked by 2 % agarose gel electrophoresis. Purified products were sequenced with BigDye Terminator (v 3.1) (Applied Biosystems) using an ABI 3500 XL Genetic Analyzer (Applied Biosystems). Sequences were manually checked and assembled using electropherograms with SEQUENCHER (v 4.9) (Gene Codes Corporation, MI, USA).

A set of 10 microsatellite markers were screened, of which six highly polymorphic markers (Polymorphism Information Content PIC > 0.5) were selected for genetic analyses (Table S2). The forward primer of each locus was tagged with a 5′ universal sequence tail of M13 (5′-TGTAAAACGACGGCCAGT), M13R (5′-CAGGAAACAGCTATGACC), or T7 (5′-TAATACGACTCACTATAGGG) (Ge et al., 2014), and labeled with FAM, HEX, and TET, respectively. Multiplex panels were set as per the molecular size and colour of fluorescent dye and PCRs were amplified in 10 μl volume, comprising 5 μl Multiplex Buffer (QIAGEN), 0.5 μl Q solution, 0.2 μM labeled forward primer (Applied Biosystems), 0.2 μM non labeled reverse primer, and 1 μl (~20–80 ng) template DNA. The touchdown PCR settings were: an initial denaturation (95 °C for 15 min), eight cycles of denaturation (95 °C for 45 s), annealing (58 °C for 60 s), and extension (72 °C for 75 s), followed by 15 cycles of denaturation (95 °C for 45 s), annealing (58 to 50 °C for 60 s) and extension (72 °C for 75 s), followed by 12 cycles of denaturation (95 °C for 45 s), annealing (52 °C for 60 s) and extension (72 °C for 75 s), ending with a final extension (60 °C for 30 min). Each experiment included both positive and negative controls. The alleles were resolved using Liz-500 Size Standard (Applied Biosystems) in an ABI 3500XL Genetic Analyzer (Applied Biosystems) and electropherograms visualized with GeneMarker (v 2.7.4) (Applied Biosystems). Three independent scorings were done to minimize the chances of genotyping errors. MicroChecker (v 2.2.3) (Van Oosterhout et al., 2004) was used to check for the presence of null alleles and GenAlEx (v 6.5) (Peakall and Smouse, 2006) for Hardy-Weinberg equilibrium (HWE).

2.3. Mitochondrial analyses

2.3.1. Phylogeny and genetic diversity

The contig sequences of Cyt *b* (776 bp) and ND4 + tRNA (902 bp) were manually checked and concatenated (1678 bp). We incorporated previously published sequences (Vamberger et al., 2020) from NCBI GenBank (Table S1) to obtain nearly range-wide coverage. The final dataset was aligned using Clustal W (Thompson et al., 1994) in BioEdit (v 7.1.3) (Hall, 1999). To infer phylogeny, we used Bayesian Inference (BI) in BEAST (v 1.7) (Bouckaert et al., 2019) to build a single consensus tree with the Burmese star tortoise (*Geochelone platynota:* Acc. No. LR697083 and LR697084) as the outgroup. The evolutionary best-fit model (HKY + G) was determined with Bayesian Information Criterion (BIC) in MEGA 11 (Tamura et al., 2021). The Markov Chain Monte Carlo (MCMC) was run for 10 million generations, sampling per 1000 generations and 10 % burn-in. The convergence and Effective Sample Size (ESS) was checked in Tracer (v 1.7) (Rambaut et al., 2018) and the consensus tree was visualized in FigTree v 1.4.4 (Rambaut, 2017). To measure genetic diversity across multiple indices, DnaSP (v 6) (Rozas et al., 2017) and Arlequin (v 3.5) (Excoffier and Lischer, 2010) were used to compute the number of Singleton variable sites (S), Parsimony informative sites (P), number of haplotypes (H), the haplotype (Hd) diversity, nucleotide diversity (π), Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989). To understand the spatial distribution of haplotypes, a haplotype network was constructed using the median-joining (MJ) method in PopART (v 1.7) (Leigh and Bryant, 2015).

2.3.2. Genetic distance and differentiation

Genetic distance was measured using three approaches. Firstly, traditional uncorrected p-distances and the best-fit model-based methods were implemented in MEGA 11 (Tamura et al., 2021) to estimate the differentiation in ISTs compared to the sister taxa, the Burmese

star tortoise. Secondly, an analysis of molecular variance (AMOVA) in Arlequin (v 3.5) (Excoffier and Lischer, 2010) was used to assess the proportion and distribution of genetic variance in IST populations through pairwise genetic divergence using the fixation index (F_{ST}).

Lastly, we used the Bayesian approach to delineate the phylogenetically retrieved clades into hierarchical taxonomic boundaries, assessing whether the genetic differentiation was taxonomically significant. The Bayesian Poisson Tree Process (bPTP), a coalescence-based species delimitation test was used to validate the taxonomic significance of the phylogenetic subdivision. bPTP uses Bayesian support to identify putative species boundaries in the phylogenetic tree through the number of nucleotide substitutions among haplotypes elucidating inter and intraspecific patterns. The MCMCs were run for 10^4 generations with 10 % burn-in, and convergence was visually checked for reliability.

2.3.3. Divergence dating and historic demography

The divergence time among genetic lineages of IST was estimated using a Strict Clock model in BEAST (v 2.5) (Bouckaert et al. 2019). We included homologous sequences from NCBI of additional turtle species representing families Testudinidae and Geoemydidae (Table S3). Two nodes, Testuguria (~103 Ma with 95 % HPD: 95–111 Ma) and Testudinidae (~83 Ma with 95 % HPD: 76–90 Ma) were used to calibrate the phylogeny based on extensive and comprehensive studies of fossil records and multiple markers (Selvatti et al., 2023). The tree prior was set to the Yule speciation model. The MCMCs were run for 10 million generations and logged per 1000 generations, with the first 10 % discarded as burn-in. Tracer (v 1.7) (Rambaut et al., 2018) was used to validate the runs' convergence and TreeAnnonator (Drummond and Rambaut, 2009) to retrieve the tree with maximum credibility. The resultant phylogenetic tree was visualized in FigTree (v 1.4.4) (Rambaut, 2018).

Temporal demographic changes in effective population size were inferred with Coalescent theory-based Bayesian Skyline Plots (BSP) (Drummond et al., 2011) in BEAST (v 2.5) (Drummond et al. 2019). We used a Strict Clock model with previously studied mutation rates (1.80e−07) in related tortoise species (Jensen et al., 2018), running 100 million steps, sampling every 10,000 steps, discarding the first 10 % as burn-in. The convergence was verified from MCMC traces in Tracer (v 1.7) (Rambaut et al., 2018).

2.4. Microsatellite analyses

2.4.1. Population structure

The number of genetic units in IST was examined using Bayesian clustering analyses in STRUCTURE (v 2.3.4) (Pritchard et al., 2000). The likelihood estimates for the number of homogenous genetic clusters (K) based on relative contribution from each individual was assessed without a priori knowledge of the individual's geographic origin. An admixture model was used with correlated allele frequency and a burn-in of 100,000 MCMCs. Ten independent runs were carried out corresponding to K: 1 to 10 and were visualized in ClumpaK (Kopelman et al., 2015). The most optimal value of K representing the most statistically robust number of clusters was estimated from delta K with STRUCTURE HARVESTER (Earl and VonHoldt, 2012) and Evanno's method (Evanno et al., 2005).

The findings of STRUCTURE were supplemented with the model-free and multivariate-based Discriminant Analysis of Principal Components (DAPC), which transforms the data using Principal Component Analysis (PCA) followed by identification of genetic clusters using Discriminant Analysis (DA). DAPC was performed and visualized using the ADEGENET package in R (Jombart, 2008).

2.4.2. Genetic diversity and divergence

The genetic variation within each group identified by STRUCTURE was assessed in GenAlEx (v 6) (Peakall and Smouse, 2006) using observed (H_o) and expected heterozygosity (H_e). The genetic divergence between and within the groups was estimated with pairwise F_{ST} (Weir, 1984) along with mean inbreeding coefficient (F_{IS}) and allelic richness *AR* in FSTAT (v 2.9.3.2) (Goudet, 2002).

2.5. Morphometry

The morphometric differentiation between the northwestern and southern groups was analyzed based on seven morphometric measurements. The characters were selected based on previous studies (Bonnet et al., 2001; Xiao et al., 2023) on Testudines and straight line measurements were taken to the nearest 0.1 mm using digital calipers: Straight Carapace Length (SCL), Straight Plastron Length (SPL), Straight Carapace Width (SCW), Straight Plastron Width (SPW), Carapace Height (CH), Anal Scute Length (ASL), and Anal Scute Width (ASW). Since ISTs exhibit sexual dimorphism and relative age estimation can be done through carapacial scute rings, for each individual, sex and approximate age were also recorded. The exploratory analyses showed a distinct variation among sexes and ages in terms of size; hence, further analyses were categorized accordingly. For adult ISTs (≥7 years or SCL 124 mm), Principal Component Analysis (PCA) was carried out using the statistical package (prcomp) in R. Consequently, distinct PCAs were executed for discrete age and sex subgroups.

3. Results

3.1. Phylogeny and genetic diversity

The 82 concatenated mtDNA sequences clustered into two genetically distinct groups: Northwestern Group (NG) and Southern Group (SG) (Fig. 2). The SG was further subdivided into Southern Indian (SI) and Sri Lankan (SL) sub-groups. The SI sub-group comprised multiple lineages corresponding to the biogeographic landscapes across the species' range: Eastern Ghats (EG), Deccan plateau (DP), Nilgiri Hills (NH), and Coromandel Coast (CC). We found clear genetic structuring in the mtDNA with lineages corresponding to their geographic landscape (Table S3). Consistent genetic signatures were observed corresponding to the major clusters, NG, and SG and the sub-clusters in the SG in Cyt *b* and ND4. The Cyt *b* gene showed two SNPs differentiating between SG and NG (14,988: NG(T)/SG(C) and 15,022: NG(G)/SG(A)), and three SNPs specific to southern sub-clusters Deccan Plateau (15,040: C), Nilgiri Hills (15,061: T) and Coromandel Coast (15,064: T). The ND4 gene showed four SNPs differentiating between SG and NG (11,010: NG(A)/SG(C); 11,019: NG(G)/SG(A); 11,031: NG(G)/SG(A) and 11,079: NG(G)/SG(A)).

In the comprehensive dataset of ISTs, a total of 30 haplotypes were identified (Cyt *b*: 19; ND4 with tRNA: 27). This dataset exhibited 12 segregating sites, consisting of 22 Parsimoniously Informative sites and Singleton sites. The haplotype diversity (Hd) of 0.91 ± 0.02 was estimated, while the nucleotide diversity (π) was 0.00323. Intriguingly, the genetic diversity within the SG was higher than NG across all the indices (Table 1 and Fig. 4). Notably, the distribution of haplotypes spatially correlated with the phylogenetic relationships evident among the diverse groups and sub-groups. The MJ network also showed two major clusters corresponding to the phylogenetic clades: NG and SG, with significant divergence by six mutational steps (Fig. 3). However, there were relatively fewer substitutions separating sub-groups within the major groups, indicating genetic coherence within groups.

The genetic diversity of ISTs was calculated using the six microsatellite markers with a mean PIC (0.72) (Table S2) genotyped across 82 samples (NG = 38 and SG = 44). A mean number of 8.67 ± 1.73 alleles were identified, with the number of alleles per locus ranging from 4 to 17. No significant allelic dropout was detected at any locus, but a low incidence of null alleles was observed in the SG, which was assessed after the Bonferroni correction. All the loci were found to be significantly deviating from HWE ($P < 0.001$) without any linkage disequilibrium ($P < 0.05$).

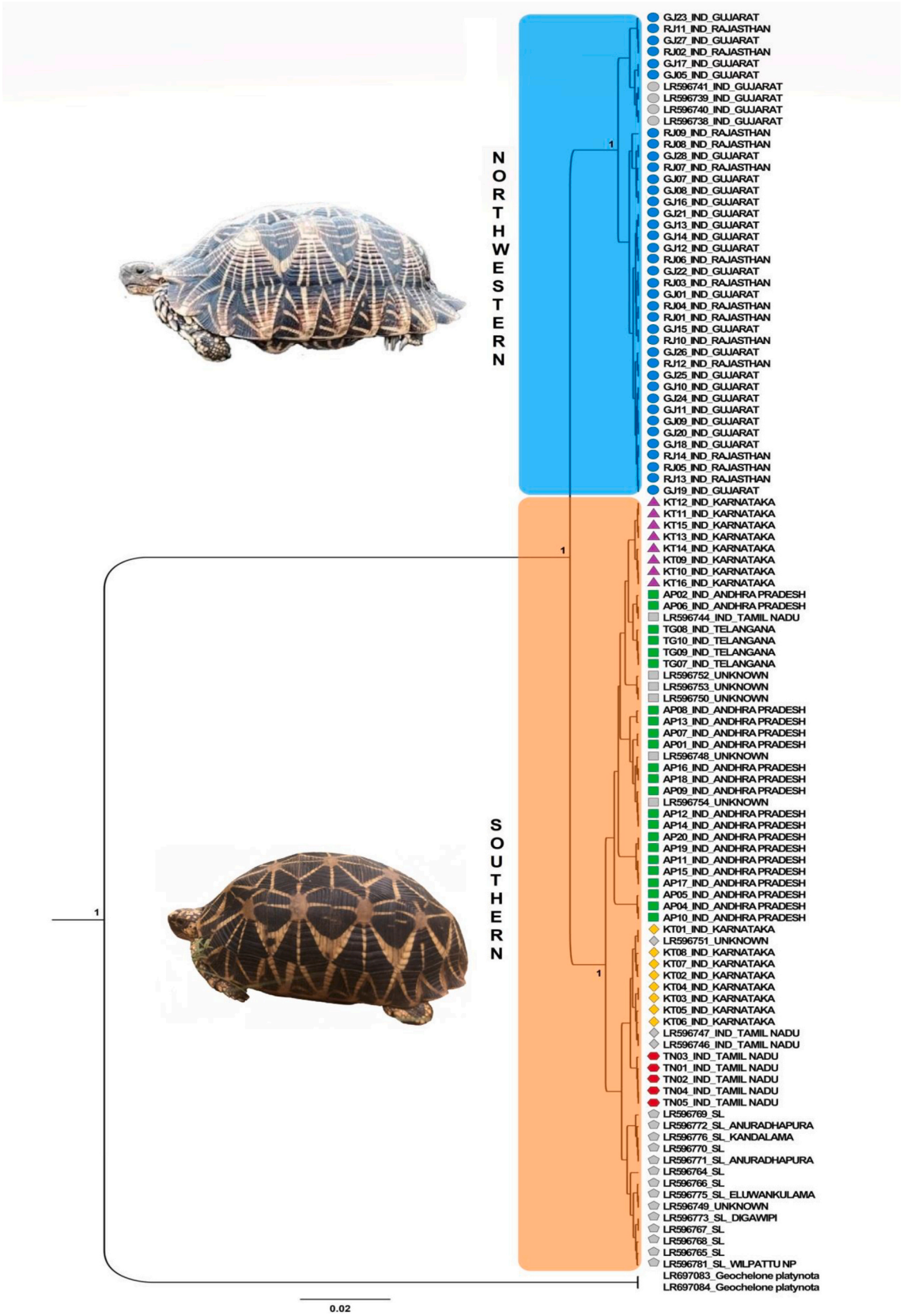

Fig. 2. Bayesian phylogenetic tree for IST based on concatenated mitochondrial regions (Cyt b + ND4 and adjacent tRNA). Samples collected in this study have symbols with colours corresponding to sampling landscapes/lineages while sequences from Vamberger et al. (2019) are denoted in Grey corresponding to shapes as per reference landscape/lineages: NG (Blue circles), DP (Purple triangles), EG (Green squares), NH (Yellow diamonds), CC (Red hexagons) and Sri Lankan sequences (Grey pentagons). Numbers next to nodes indicate their respective posterior probability (PP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Estimates of genetic diversity indices and population demography tests in IST populations based on mtDNA and microsatellites, with standard deviation. N = no. of samples, Variable sites (Singleton sites/Parsimony Informative sites), H = no. of haplotypes, Hd = Haplotype diversity, π = Nucleotide diversity; n = no. of samples, Ar = Allelic Richness, H_o = Observed Heterozygosity, H_e = Expected Heterozygosity, F_{IS} = Fixation Index; *non-significant values with P-value > 0.01.

Population	mtDNA diversity indices							Microsatellite diversity indices				
	N	Variable sites	H	Hd	π	Tajima's D*	Fu's Fs*	n	Ar	H_O	H_E	F_{IS}
Northwestern India	42	6 (2/4)	7	0.54 ± 0.08	$4.7*10^{-4}$	−0.54	−0.20	38	6.33 ± 2.20	0.29 ± 0.07	0.51 ± 0.13	0.38 ± 0.15
Southern India	67	22 (10/12)	23	0.95 ± 0.01	$16.3*10^{-4}$	−0.29	0.44	44	11 ± 2.5	0.44 ± 0.05	0.78 ± 0.04	0.42 ± 0.08
Overall	109	34 (12/22)	30	0.91 ± 0.02	$32.3*10^{-4}$	−0.35	0.30	82	8.67 ± 1.8	0.37 ± 0.05	0.65 ± 0.08	0.38 ± 0.08

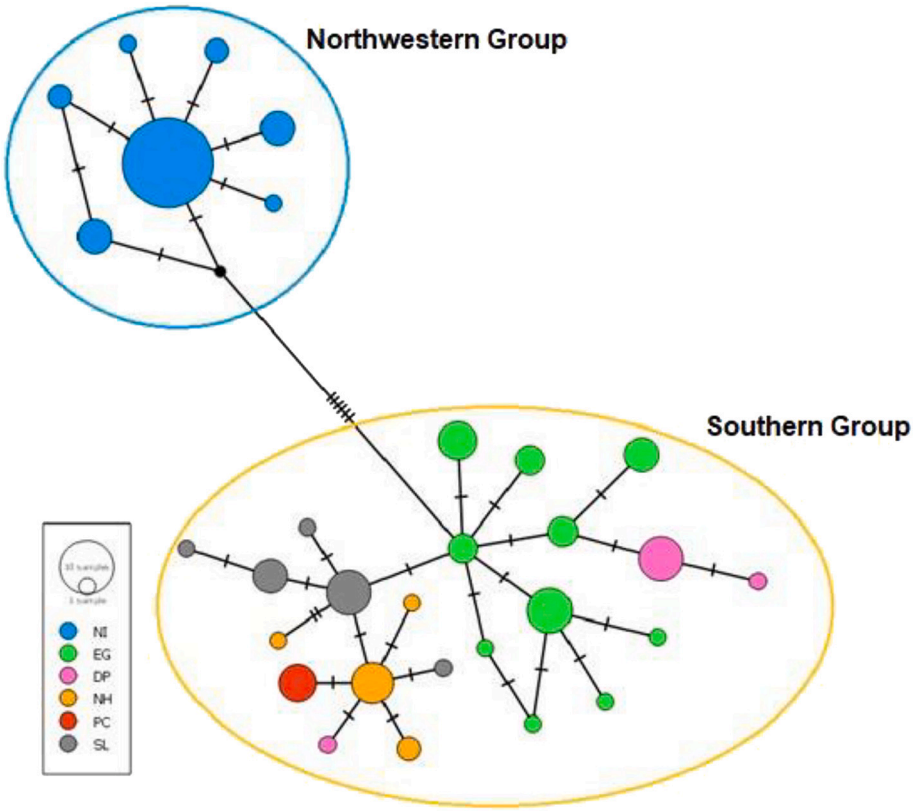

Fig. 3. IST (*G. elegans*) haplotype network based on medium joining method for the 1678 bp concatenated mtDNA fragment (Cyt *b* + ND4 with adjacent tRNA). Circles represent individual haplotypes and sizes reflect the relative abundance of respective haplotype.

Microsatellite diversity indices were higher in the SG than the NG, a pattern similar to mtDNA. Allelic richness (*AR*) was higher in the SG population (11 ± 2.5) as compared to NG (6.33 ± 2.20). The observed heterozygosity and expected heterozygosity were higher in SG (H_o: 0.44 ± 0.05; *He*: 0.78 ± 0.04) than in NG (H_o: 0.29 ± 0.07; *He*: 0.51 ± 0.13) (Table 1). The mean inbreeding coefficient (F_{IS}) value for all the IST were greater than zero (between 0.38 and 0.42), indicating a deficiency of heterozygotes (Table 1), which may be attributed to Wahlund effect and population not being in HWE.

3.2. Population structure and differentiation

The mtDNA based AMOVA indicated high differentiation (F_{ST} = 0.80) between the NG and SG with statistical significance $P < 0.001$. Of the total variation, the maximum variation was among the groups (64.88 %), followed by among populations within groups (15.30 %) and the minimum variation within populations (19.82 %) (Table 2). The estimated evolutionary distance over sequence pairs between groups indicated the mean distance between the NG and SG to be 0.5 % and 0.7 % in Cyt *b* and ND4 + tRNA, respectively. The genetic distance between the NG and the Burmese star tortoise was 4.9 % and 4.4 % in Cyt *b* and ND4 + tRNA, respectively. The uncorrected p distance between the NG and the SG ranged from 0.3-0.6 % in Cyt *b* and 0.6-0.8 % in ND4 + tRNA, while between the NG and Burmese star tortoise, it was 4.5 % and 4.3 % in Cyt *b* and ND4 + tRNA, respectively (Tables S4 and S5).

The microsatellite based Bayesian clustering in STRUCTURE identified a clear structure at K = 2, corresponding to the highest value of the delta (K) and likelihood values (Figs. 5 and S1). The two clusters differentiated the NG individuals from the SG as two distinct genetic groups. However, DAPC not only corroborated the presence of two major distinct groups (NG and SG) but further distinguished four genetic sub-lineages in the SG corresponding to geographic clusters: DP, EG, NH, and CC, while NG remains a singular lineage (Fig. 5). The pairwise F_{ST} between the NG and the SG was significantly high (F_{ST} = 0.14), indicating high differentiation between groups. The bPTP approach with MCMC convergence suggested two putative taxa, one corresponding to the NG and the other to the SG (Fig. S2). Thus, the nuclear differentiation and population structure analysis results were congruent with the phylogenetic, haplotype network, and differentiation analysis results from the mitochondrial dataset.

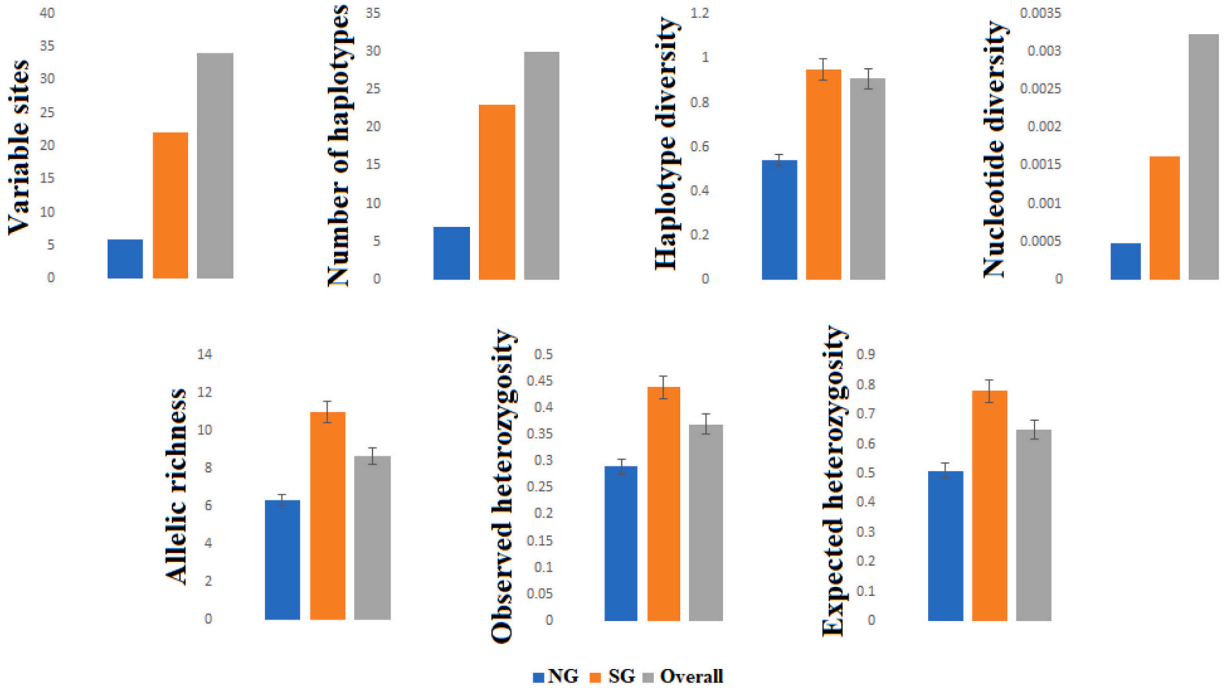

Fig. 4. Genetic diversity in IST based on mtDNA (top row) and microsatellites (bottom row). The three colour-coded columns represent the NG (blue), the SG (orange), and the Overall (grey) diversity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

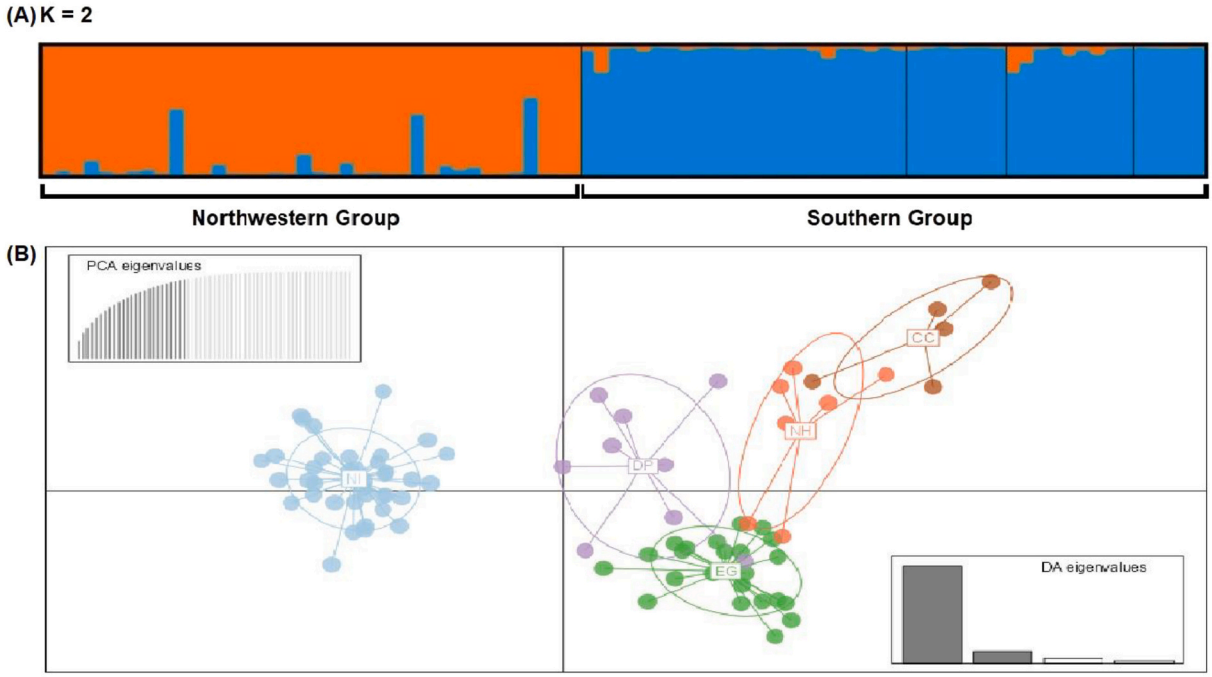

Fig. 5. (A) Structure based population assignment for the microsatellite dataset of samples from India. Bar plot for K = 2 inferred clusters with each vertical line representing an individual genotype for the 6 polymorphic loci and each colour corresponding to a genetic group. (B) DAPC scatter plot showing genetic clustering with different populations denoted by different ellipses and colours: Northwestern Group (NG): blue, Deccan Plateau (DP): purple; Eastern Ghats (EG): green; Nilgiri Hills (NH): orange and Coromandel Coast (CC): brown; the PCA and DA eigen values are indicated in the top left and bottom right, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Divergence dating and demographic pattern

The divergence time estimates dated the root age of Testuguria (Time to the Most Recent Common Ancestor (TMRCA) of Testudinidae and Geoemydidae) to 103 Ma with (95 % HPD: 95–111) and Testudinidae to 83 Ma (95 % HPD: 76–90). The split between *Centrochelys* (sister taxa to *Geochelone*) and *Geochelone* occurred around 43 Ma (95 % HPD: 35–51) and the split between the *G. platynota* and *G. elegans* around 11.5 Ma (95 % HPD: 8.5–15), indicating the latter as the older *Geochelone* lineage. In *G. elegans*, the NG diverged from the SG around 2 Ma (95 % HPD: 1–3),

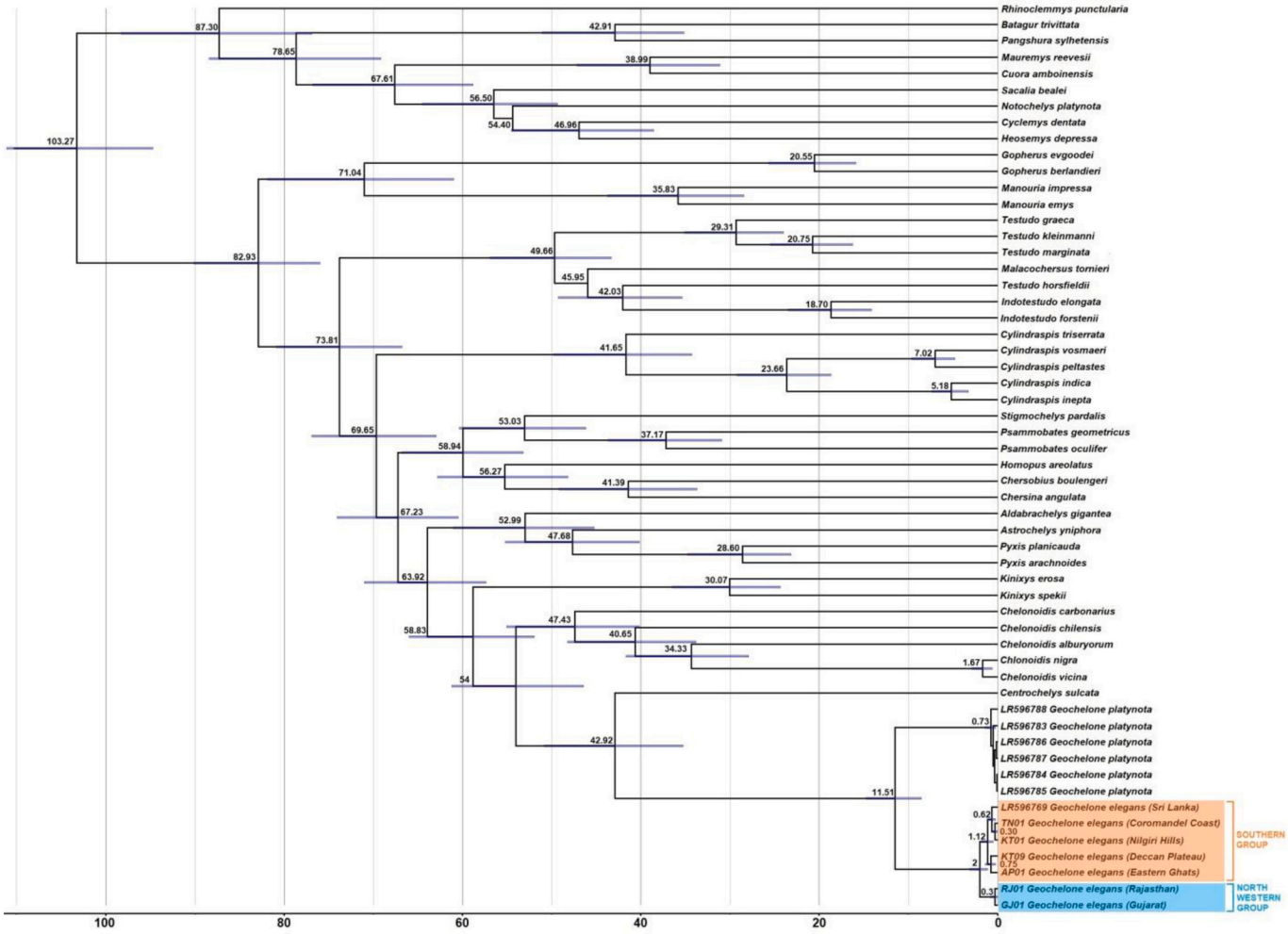

Fig. 6. Divergence dating based on maximum credibility tree using the concatenated dataset (Cyt *b* + ND4 with adjacent tRNA). The x-axis represents time in million years.

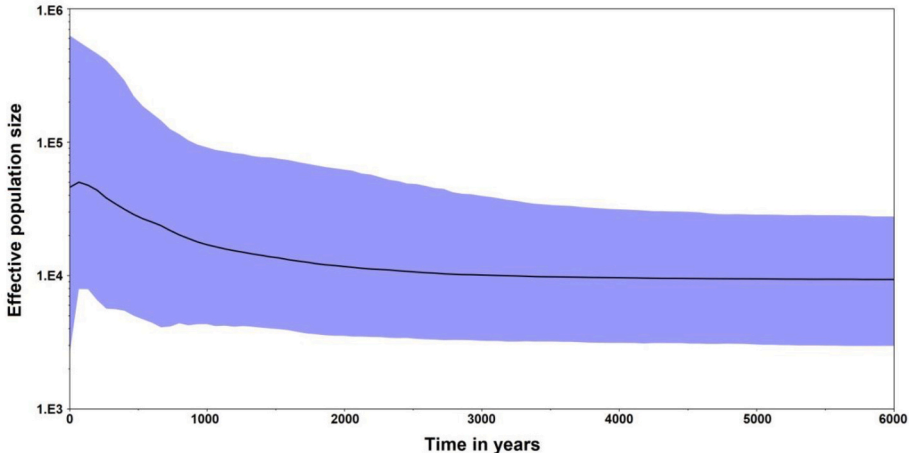

Fig. 7. Bayesian skyline plot denoting changes in population demography of IST with time.

indicating the SG to be the older lineage of IST (Fig. 6).

The neutrality tests to check for contemporary evidence of population expansion were not significant: Tajima's D test was negative for both NG and SG, while Fu's FS test was positive for both NG and SG (Table 1). The BSP with significant ESS (>200) and MCMC convergence indicated that the effective population size (Ne) of ISTs has been historically stable, followed by a steady expansion since the last 2000 years. However, a recent and slight decline began around 100–120 years ago (Fig. 7).

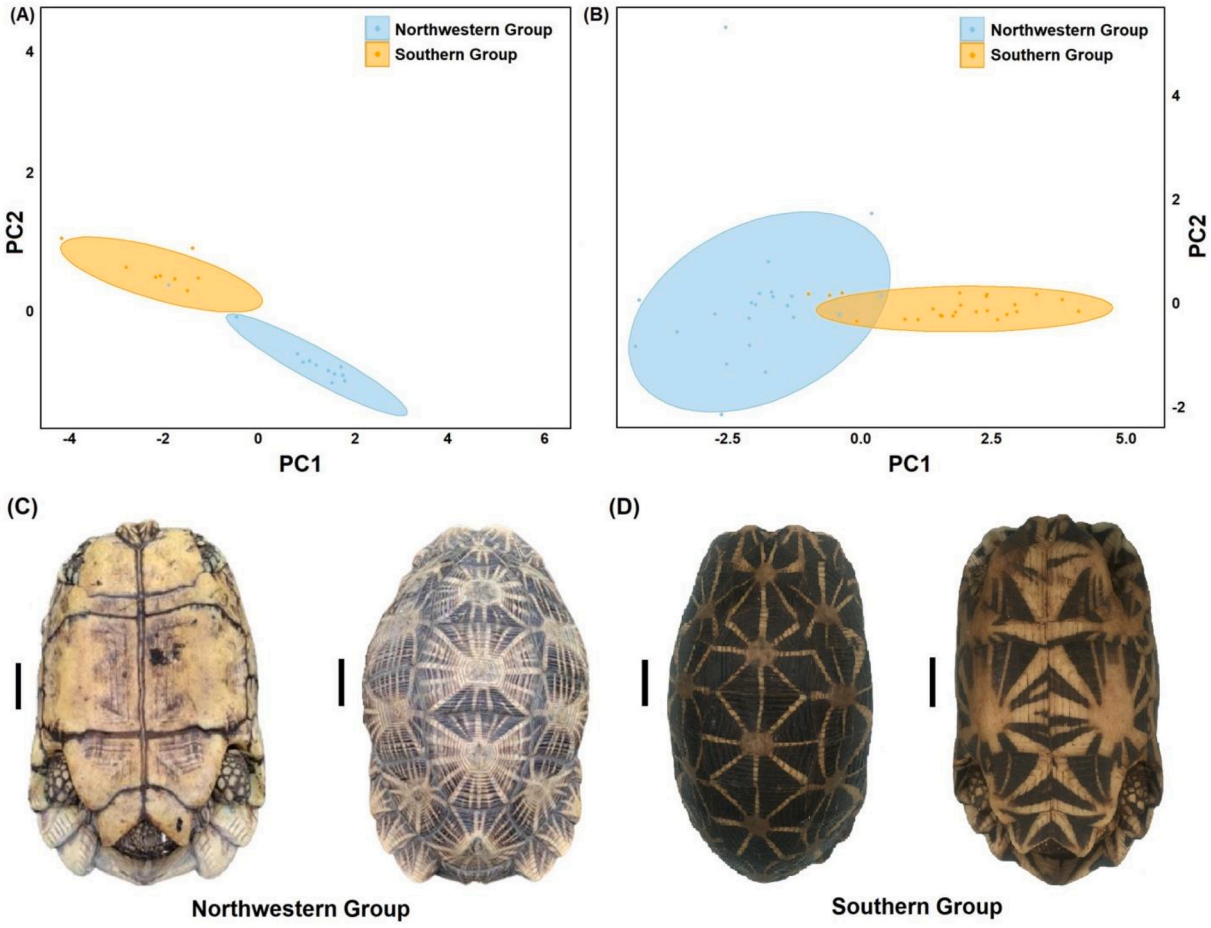

Fig. 8. Principal Component Analysis (PCA) using morphometric data for the two groups of ISTs in adult (A) Females and (B) Males. The individuals from Northwestern Group are represented in Blue colour, while the individuals from Southern Group are represented in Orange. (C) and (D) show Dorsal and Ventral view of ISTs from Northern and Southern Group, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analysis of the Molecular Variance (AMOVA) table statistics. Abb.: *df* = degree of freedom, *SS* = Sum of Squares, *MS*: Mean Squares, % = percentage of genetic variation; *significant *P*-values < 0.005.

Source of variation	df	SS	Variance*	%
Among groups	2	196.805	2.47871	64.88
Among populations within groups	5	36.121	0.58452	15.30
Within populations	103	77.992	0.75721	19.82
Total	110	310.919	3.82044	100.00

3.4. Morphometry

As previously reported, adult females are indeed larger than adult males, but we also observed an interesting sex and size gradient that differed between the two groups in India: $NG_{Female} > NG_{Male} \approx SG_{Female} > SG_{Male.}$ Adult females in both groups were larger than their male counterparts across all parameters (SCL, SPL, SCW, SPW, CH, and ASW) except one (ASL) (Fig. S3).

Separate PCAs of adult females and males revealed two distinct clusters corresponding to the respective geographic populations, i.e., NG and SG India (Fig. 8). The PC 1 and 2 together accounted for 96 % and 86 % of the total variation in females and males, respectively (females: PC1: 74 %, PC2: 22 %; males: PC1: 73 %, PC2: 13 %). While the variation in females was primarily driven by CH, SCL, and PL with a positive correlation, the variation in males was majorly driven by CH, SCL, and PW with a negative correlation (Table S7).

4. Discussion

Contrary to previous studies and widely believed notions, the results identified a geographically clear genetic structure in the ISTs in the wild despite decades of illegal poaching and unscientific release. A thorough sampling across India enabled us to improve the resolution and insights into the evolutionary history of the highly traded species, enabling its effective management based conservation. We confirm the presence of a clear genetic structure, as also suggested by Gaur et al. (2006) but rather than three allopatric groups (Northern, Southern, and Sri Lankan IST), our results identify two major groups: the Northwestern group and the Southern group, with the Sri Lankan lineage as a sub-division within the Southern Group. In contrast to Vamberger et al. (2020), which suggested erosion of phylogeographic signature, we found unambiguous geographic signatures including from previously unsampled locations. The data from Vamberger et al. (2020) also clustered with the expected clades in our analyses, supporting the findings. However, to focus on the genetic status in the wild population, as a precaution, we did not include two groups of sequences from Vamberger et al. (2020) that showed unusual clustering with high mutation rates in the preliminary phylogenetic and haplotype analyses. Firstly, the sequences from Pakistan (LR596654-LR596660) clustered with sequences from SG instead of geographically closer NG, both in our analyses and the original study. This clustering with SG is ecologically unexplainable. Moreover, with the Sindh province in Pakistan being a trading hotspot, the release of traded individuals from SG in the area cannot be completely ruled out

(Ilyas, 2014a, 2014b; Khan, 2014; "Sindh, Punjab hub of illegal turtle trade", 2015; Ilyas, 2017). The four sequences from Sri Lanka (LR596672, LR596675–77) also formed a distinct cluster apart from the rest of the Sri Lankan sequences, which may be attributed to the release of seized individuals with divergent genetic makeup in the wild (Rodrigo and Prava, 2022; Gupta, 2023).

In contrast to Kundu et al. (2022), which indicated the erosion of genetic diversity, we found high genetic diversity in the SG and moderate in the NG. The disparity in outcomes could be attributed to the earlier studies relying on samples sourced from zoos, captive environments, or pet stores. Our study provides insights into the wild population of ISTs using extensive sampling throughout the range in India, building upon the findings of the previous studies. We discuss the findings of our study in a broader evolutionary context with practical implications in managing the highly trafficked species towards its long-term conservation.

4.1. The northwestern and southern IST are evolutionarily distinct

The NG and SG are the major genetic units identified through an integrative genetic and morphometric framework. While the NG represents an insular and relatively less diverse lineage, the SG is a basal, highly diverse lineage with multiple sub-lineages. Interestingly, further genetic sub-divisions within the SG were not identifiable through species delimitation, STRUCTURE, and morphometry, indicating their relatively recent evolutionary sub-division. The genetic differentiation between the two IST groups qualify the thresholds of sub-species level differentiation as followed in several other Testudines such as *Testudo* subspp., *Chelonoidis* subspp., and *Aldabrachelys* subspp. based on AMOVA, F_{ST}, and uncorrected p-distance (Fritz et al., 2009; Fritz et al., 2012; Graciá et al., 2017; Vargas-Ramírez et al., 2010; Vamberger et al., 2020). A nuanced morphological gradient between the NG and SG exists due to the interplay of sexual dimorphism and geographic adaptations. The strong genetic distinction indicates historical divergence, while their evolution in two disjunct habitats may have led to local adaptations culminating into distinct morphology.

The *Geochelone* group split from its sister taxa *Centrochelys* in the African dry zone around 43 Ma during Eocene and radiated to the Indian subcontinent via the biotic ferry model upon the Indian-Eurasian collision around 55 Ma (Abrajevitch et al., 2005; Kent and Muttoni, 2008; Datta-Roy and Praveen Karanth, 2009). The spread of *Geochelone* in the Indian subcontinent may have been enabled by the Eocene-Oligocene global shift towards a drier climate, as observed in other reptilian and amphibian species (Gower et al., 2016; Deepak and Karanth, 2018; Lajmi and Karanth, 2020). The *Geochelone* group split into an older *G. elegans* in the Indian subcontinent, and a younger *G. platynota* in the bordering South-East Asia (present day Myanmar) around 11.5 Ma during Miocene. The current natural habitats of ISTs may have developed with the intense aridification in the late Miocene, which enabled the spread of open grasslands in the Northwestern Indian subcontinent and the dry zone in the peninsula, both of which are the current natural habitats to the species (Molnar and Rajagopalan, 2012; Karanth, 2003). The initial spread of C4 or savanna grassland was accompanied by increased monsoonal seasonality in the Asian subcontinent, which eventually culminated in the expansion of C4 vegetation in the Indian peninsula during the Plio-Pleistocene (Zhisheng et al., 2001; Edwards et al., 2010; Lajmi and Karanth, 2020; Dunlea et al., 2020). The retreat of humid forests to patches across Southwest India and Sri Lanka (Deepak and Karanth, 2018) coincided with the split in *G. elegans* group into an older and basal stock of SG and the younger NG around 2 Ma, allowing the isolation of dry zone adapted lineages. The resultant higher diversification in the SG (across Southern India and Sri Lanka) comprises sub-groups with relatively recent (~1–0.3 Ma) evolutionary origin. This divergence dating is in sync with the phylogenetic clustering in ISTs where the southern sub-groups diverged as two separate clades, the upper peninsular clades (EG and DP) and the lower peninsular clades (NH, CC, and SL), around a million years ago.

The current distribution range of the IST in the Indian subcontinent thus represents the refugia population of the species rather than distribution through diversification or range extension. Historically, the population demography of ISTs has been stable, as suggested by the neutrality tests and corroborated by the BSP, which suggested a past stabilization, followed by a steady expansion around 2000 years ago and a recent declining trend since the last ~100–120 years.

4.1.1. Northwestern Group

The landscape inhabited by the NG represents the northernmost extension of the peninsular landmass. The habitat ranges from arid desert to semi-arid savannas comprising the Thar desert to the Central zone bound by the Aravalli in the north to the Kutch and Kathiawar peninsula in the south (D'Cruze et al., 2018). The northwestern habitat is relatively homogenous, with large stretches of peneplains as compared to Southern India. This along with the evolution of the group as an insular lineage isolated due to refugial origin, may have supported the fixation of alleles adapting to the typical local habitat, resulting in the significantly moderate to low genetic diversity. Similar instances of highly localized adaptation to the desert landscape have been observed in other reptiles (Araya-Donoso et al., 2022). The neutrality tests to infer past population demography suggested historic stabilization or balanced selection.

4.1.2. Southern Group

The SG is spread across the dry zones in central to southern peninsular India and Sri Lanka, characterized by multiple ancient hill ranges with high landscape and climatic heterogeneity, ideal for lineage diversification (Deepak and Karanth, 2018; Agarwal and Karanth, 2015). In contrast to the NG, the SG is a genetic hotspot comprising multiple sub-lineages coinciding with landscape-level physiography, denoting endemic dry zone diversity. The identification of Southern India, a poaching hotspot (Anand et al., 2005; Auliya et al., 2016; D'Cruze et al., 2016; Traffic, 2019; Stoner and Shepherd, 2020) as a genetic hotspot highlights the significance of the landscape in designing conservation and management policies. The SG showed significantly higher genetic diversity than the NG, with the lower peninsular sub-lineages (NH, CC, and SL) as the ancestral stock evolving closely together compared to the upper peninsular sub-lineages (EG and DP).

The SL sub-lineage as the southernmost distribution of the IST being a part of the basal southern ancestral unit is not surprising as the landscape itself was part of the peninsular mainland during the late Pleistocene and continues to be part of the Deccan plate (Mani, 1974). While the CC sub-lineage represents the southernmost distribution of IST in India, closest to the SL sub-lineage, the NH is where the Eastern and the Western Ghats meet and represents the sub-lineage in the borders between the states of Karnataka and Tamil Nadu. Northwards, The EG on the eastern side of the peninsula holds a sub-lineage inhabiting the states of Telangana and Andhra, which is also the northernmost distribution of the SG, while the DP represents a central peninsular sub-lineage in Karnataka, situated between the Eastern and Western Ghats.

The evolution of spatial genetic variation in habitat specialists such as the ISTs has been thus driven by aridification and the spread of grassland ecosystems. Similar events of diversification through endemic radiation aridification in peninsular India have been observed in other reptiles such as geckos, *Ophisops*, and *Sitana* (Lajmi and Karanth, 2020; Agarwal and Ramakrishnan, 2017; Deepak and Karanth, 2018). The contrast in diversity and diversification between the NG and SG of ISTs is driven by an interplay of historical and ecological factors, primarily the precipitation regime. The SG represents the ancestral stock in a highly heterogeneous landscape, ripe for genetic diversification, while the NG represents the refugia offshoot of the species, which has adapted itself to a highly specific habitat, leading to the fixation of locally adapted genes. The deep historical divergence adapted to local ecology has resulted in the present distribution and distinction between the two groups of ISTs,

which must be accounted for in the management strategies to conserve the natural genetic and adaptive potential of the highly trafficked species.

4.2. Conservation and management issues

The CITES CoP 18, 2021, supported by the range states, has identified the major challenges towards successful handling of live seizures, which is the most pertinent management issue in the species: (1) lack of national policies, (2) incomplete understanding of the role of various national agencies, (3) lack of sufficient financial resources, and (4) lack of skilled staff.

Despite increasing success in intercepting large seizures, the primary hurdle in the management of ISTs is the lack of evidence-based release and rehabilitation. Genetic evidence can be the guiding principle, as morphology alone is inconclusive in determining the provenance of the large number of juveniles and sub-adults primarily targeted in the illegal pet trade. In the absence of a science-based management policy, many seized ISTs have been released outside their native ranges, endangering novel habitats with pathogens and threatening local population-specific signatures in the species' natural habitat due to intermixing with individuals from other areas (de Silva et al., 2017). Despite well-meaning intentions, this impedes responsible rehabilitation of the seized tortoise and the source population suffers from incremental loss of gene pool. Recent studies on trafficked species such as the Olive Ridley turtle and Galapagos tortoise have demonstrated the application of comprehensive genetic characterization as an effective tool against IWT (Stelfox et al., 2020; Quinzin et al., 2023). The case of ISTs presents a suitable conservation issue that can be made simpler with genetic tools guiding management decisions, harnessing the natural evolution of the species towards combatting its IWT.

For efficient handling of seizures, collaboration among national and regional agencies must be institutionalized with procedural guidelines. The absence of national legislation for the protection of exotic wildlife in most of the transit hubs and destination states, especially in South and South East Asia, hinders the alignment of conservation efforts on a larger scale (Stoner and Shepherd, 2020). Recently, Malaysia, a major destination state, accorded legal protection to ISTs, effectively making the species' presence in pet stores illegal (Stoner and Shepherd, 2020). Similar coordinated legislations across states at various stages of trade networks must be strictly enforced with implicit flexibility for fast implementation of international rehabilitation efforts, such as the seizure in Singapore studied by Gaur et al. (2006) for release into native habitats in India. A concerted effort integrating genetic intelligence with the vigilance of customs officials and wildlife agencies at borders, complementing monitoring NGOs, can consolidate scarce resources like skills and finance and strengthen the conservation of highly trafficked species (CITES, 2019).

4.2.1. Genetically guided management recommendations

Our study provides comprehensive data on wild IST populations, uncovering hither to unknown geographic signatures that can be used to inform management decisions on release and rehabilitation and monitoring trafficking and trade in the species. India, home to the largest natural population of ISTs, the biggest poaching source, and also the global hotspot for seizures, can take the lead in policy-based scientific management of highly trafficked species by institutionalizing a national policy guided by evidence-based release and rehabilitation. The guidelines may be defined for different categories of states: native ranges (where the species naturally occurs) and trade routes or destination states (where the seizure occurs). It would ensure the protection of the species in states, whether native or exotic, in distribution. In case the seizure occurs outside the native ranges, the species must be rehabilitated into a genetically related population, and multiple destination states in India can be considered as several natural populations of ISTs, as identified in the study, inhabit landscapes cutting across political boundaries. Release of seized ISTs in not native ranges should be avoided and the priority should be to rehabilitate the seized tortoises in a genetically similar population, enriching the gene pool of vulnerable populations. Southern India being both a poaching and a genetic hotspot for the species, the database can guide the management of seizures in the landscape, considering the presence of release sites such as the Chinnar WLS and the Sathyamangalam TR with the risk of admixture. There can be a larger collaborative effort comprising areas with a significant presence of ISTs to ascertain the origin of released ISTs at various release sites and zoos to understand the broader pattern of the current presence of the species, both in and outside their native habitats. Larger robust networks of various enforcement agencies and NGOs can be harnessed to effectively use collective intelligence.

The naturally deep historical divergence in the two IST groups should be maintained to minimize the impact of genetic admixture due to anthropogenic factors such as illegal trade and release. Care must be taken to release seized tortoises in their closest genetic landscape based on the reference database of wild ISTs and not intermix the NG and the SG. The two groups in the highly trafficked species represent distinct Evolutionarily Significant Units with deep genetic divergence, which manifests morphologically. This qualifies the groups to be treated as distinct subspecies based on their mitochondrial and nuclear DNA variations, which will enhance effective communication and awareness within and beyond the scientific community, especially in legislations and management policies for release, rehabilitation, and reintroduction (Moritz, 1994; Kindler and Fritz 2018).

To effectively manage both the IST groups, we suggest the nomenclature *Geochelone elegans elegans* for the Southern Group (SG), representing the oldest lineage of IST, and *Geochelone elegans stellata* for the Northern Group (NG). Our nomenclature adheres to the recommendations of the Turtle Taxonomy Working Group (Rhodin et al., 2021) and complies with the International Code of Zoological Nomenclature (ICZN) (ICZN, 1999). The proposed names are based on the comprehensive description provided by Gray in 1870, which highlights the distinctions between the previously designated holotypes. Gray (1870) specifically refers tothe original holotype described by Schoepff in 1792 as *Testudo elegans* (the 'fewer-rayed variety'), characterized by a relatively more blackish shield. In contrast, Gray (1870) notes the holotype described by Schweigger in 1812 as *Testudo stellata*, which features more numerous rays. These morphological descriptions align with the characteristics of the SG and NG, respectively.

The SG is relatively smaller and displays fewer rays with a more contrasting carapace, as opposed to the larger NG, which exhibits more numerous rays with a relatively less contrasting carapace (Fig. 8) (Das, 1995; Frazier, 1992; Vyas, 2006). Accordingly, the name *Geochelone elegans elegans* is proposed for the SG subspecies as the nominotypical subspecies in accordance with Article 47 of the ICZN (after the SG holotype described by Schoepff, 1792) and *Geochelone elegans stellata* for the NG subspecies in accordance with Article 23 of the ICZN, the Principle of Priority and Synonymy (after the NG holotype described by Schweigger, 1812).

We recommend regular usage of the scientific database presented in the study for future release, rehabilitation, and reintroduction programs to conserve the species by harnessing its natural genetic variation. We propose the declaration of the two groups as distinct subspecies for translating science into on-ground conservation with immediate effect on release policies at the grassroots. We also recommend updating the IUCN database on the species distribution range, adding the landscapes covered during this study along with a comprehensive characterization of other natural habitats and release sites in India, Pakistan, and Sri Lanka to correct inadvertent admixture. It would ensure that the populations inhabiting a landscape belong to the same ESU, minimizing the impact of illegal trafficking on the species and enabling it to realize its natural adaptive trajectory.

5. Conclusion

We provide insights into the population status and evolutionary background of one of the world's most trafficked species, the ISTs, bridging a crucial knowledge gap in managing and conserving the species. We highlight the presence of a clear genetic structure in wild ISTs with two distinct ESUs: Northwestern and Southern, which should be the guiding framework for all future management decisions for the release, rehabilitation, and reintroduction of seized tortoises. We identify the Southern population, coinciding with a major poaching hotspot, with high diversity and comprising multiple sub-lineages to be of special significance for conservation programs. The two groups represent historically deep divergent lineages and must be treated as separate subspecies with the name *G. e. elegans* for SG and *G. e. stellata* for NG for proper management.

The resource furnished in the study includes novel geographic signatures and mitochondrial typing, which is a cost-effective and fast tool to identify the most genetically suitable population to rehabilitate confiscations in line with the species' evolutionary history. It will minimize the admixture of genetically distinct populations and aid in recovering vulnerable populations in poaching hotspots.

CRediT authorship contribution statement

Subhashree Sahoo: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Writing – original draft. **Ajit Kumar:** Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Jagdish Rai:** Conceptualization, Supervision, Writing – review & editing. **Sandeep Kumar Gupta:** Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they do not have any competing interests that could have appeared to influence the work reported in this paper.

Data availability

The generated sequence data will be publically available in NCBI GenBank and other details are presented in the Supplementary Material.

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Appendix A. Supplementary data

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